NOTE

Metabolites from thermophilic bacteria I: *N*-propionylanthranilic acid, a co-metabolite of the bacillamide class antibiotics and tryptophan metabolites with herbicidal activity from *Laceyella sacchari*

Hirofumi Akiyama¹, Naoya Oku¹, Hiroaki Kasai², Yoshikazu Shizuri², Seitaro Matsumoto³ and
Yasuhiro Igarashi¹

The Journal of Antibiotics (2014) 67, 795–798; doi:10.1038/ja.2014.64; published online 28 May 2014

Since the discovery of gramicidin from *Brevibacillus brevis* in 1940,¹ many important drugs including antibiotics, immunosuppressants, antidiabetic, antiobesity and anticancer drugs have been developed from the metabolites of mesophilic soil bacteria.² However, as a reflection of worldwide efforts exerted over the years, discovering new structures is becoming more and more difficult. Because expanding structural diversity of our chemical reservoir is a key requirement to the successful drug discovery research, untapped microbes are now gathering significant attention as new biomedical resources.^{3,4}

The family *Thermoactinomycetaceae* (phylum *Firmicutes*) is one of the kinships of *Bacillaceae* but has long been cataloged into *Actinomycetales* before molecular phylogenetics was available.⁵ They grow in a filamentous manner and mostly develop aerial hyphae as the typical actinomycetes do, but are clearly distinguished by forming heat-resistant spores inside cells (endospores), which is a characteristic trait of *Bacillaceae*-related taxa. The first secondary metabolite from this bacterial taxon, aromatic polyketide antibiotic thermorubin,^{6,7} was isolated at comparatively an early time in the history of microbial drug discovery, but those added during the past half century is only 10: 5'-deoxyguanosine,⁸ imidazole alkaloid sibyllimycine,⁹ an antifungal lysine derivative,¹⁰ macrocyclic thiazole/oxazole-containing cytotoxic peptides mechercharstatins¹¹ and urukthapelstatin A,¹² and acylated tryptamines bacillamides with local anesthetic^{13,14} or algicidal activity¹⁵ and their analogs (we adopt 'bacillamides' as the name for this class of metabolites following the widespread acceptance in the preceding publications, which mostly focused on algicidal bacillamide A. It should be noted that TM-64¹³ is the first to be isolated.).¹⁶ Of eleven genera currently known in this family (*Thermoactinomyces*, *Laceyella*, *Seinonella*,

Thermoflavimicrobium, *Planifilum*, *Mechercharimyces*, *Shimazuella*, *Desmospora*, *Kroppenstedtia*, *Marininema* and *Melghirimyces*, based on List of Prokaryotic Names with Standing in Nomenclature: <http://www.bacterio.cict.fr/classifgenerafamilies.html>). only two have been examined as sources for drug discovery.

As part of our program to probe the biosynthetic potential of unexploited bacterial taxa, we have conducted metabolome mining in a strain identified as *Laceyella sacchari*, which resulted in the isolation of two *N*-acylanthranilic acids (1 and 2), one of which is new to natural products, as co-metabolites of bacillamides (3 and 4) and *N*-acetyltryptamine (5).

The producing strain was isolated from a green alga of the genus *Spirogyra* collected in an irrigation ditch that runs through fallow rice fields in Toyama Prefecture, Japan. An algal piece was taken from the specimen and ground manually with a glass pestle in a sterile distilled water. After standing for 30 min, an aliquot of supernatant was spread over the surface of ISP-2 solidified with 0.7% gellan gum, and the isolation plate was incubated at 50 °C. After an overnight incubation, a colony with grayish white aerial mycelia appeared on a medium surface, which was covered mostly by mucous bacterial mass. This was picked and transferred repeatedly on Bn-2 agar until the culture became pure.

The isolate, designated IT-2L, grew well at 50 °C, but a week-long culture was necessary to see the same extent of growth at 37 °C. No growth was observed at 30 °C even after a month, indicating that IT-2L is a thermophile. The optimal growth temperature was determined to be 50–53 °C by a temperature gradient test on Bn-2 agar. Molecular phylogenetic analysis was conducted based on 16S rRNA gene sequence, which identified IT-2L as *Laceyella sacchari*.

¹Biotechnology Research Center, Toyama Prefectural University, Toyama, Japan; ²Marine Biotechnology Kamaishi Laboratory, Kitasato University, Iwate, Japan and ³Research Center, Nihon Nohyaku Co., Ltd., Osaka, Japan

Correspondence: Professor Y Igarashi, Biotechnology Research Center, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan.

E-mail: yas@pu-toyama.ac.jp

Received 27 November 2013; revised 6 April 2014; accepted 13 April 2014; published online 28 May 2014

Figure 1 Structures for 1–5.

Table 1 NMR Data for *N*-propionylantranilic acid (1) in DMSO- d_6

Position	δ_c^a	δ_H^b , mult, J in Hz, integration	HMBC ^c
1-CO ₂ H	169.3		
1	116.2		
2	141.1		
2-NH		11.14, s, 1H	3, 7
3	119.8	8.52, d, 8.0, 1H	1, 5
4	134.1	7.56, t, 8.0, 1H	2, 6
5	122.4	7.11, t, 8.0, 1H	1, 3
6	131.1	7.97, d, 8.0, 1H	1-CO ₂ H, 2, 4
7	172.0		
8	30.7	2.40, q, 7.6, 2H	7, 9
9	9.3	1.12, t, 7.6, 3H	7, 8

^a100 MHz.^b500 MHz.^cCorrelation from ¹H to ¹³C.

This bacterial species, formerly *T. sacchari*, is known as the principal cause of bagassosis, which is a type of hypersensitivity pneumonitis once prevalent among sugar mill workers.¹⁷ Intrigued by this epidemiological episode and because no secondary metabolites have so far been reported from this bacterium, we undertook metabolome mining in this strain.

Strain IT-2L was cultured in A-11M medium, and the whole culture broth was extracted with 1-BuOH. The butanolic extract was subjected to silica gel column chromatography eluted with a CHCl₃-MeOH solvent system, and fractions were analyzed by HPLC-DAD to monitor their constituents. Because most of the major constituents in the crude extract were eluted by CHCl₃-MeOH (10:1), this fraction was further separated by reverse phase HPLC to yield *N*-propionylantranilic acid (1) along with known *N*-acetylantranilic acid (2), bacillamides A (3) and B (4), and *N*-acetyltryptamine (5) (Figure 1).

The ¹H NMR spectrum (Table 1) of compound 1 was quite similar to that of 2,¹⁸ containing resonances for an *ortho*-substituted anilide core: an exchangeable proton (δ_H 11.14 s) and four mutually coupled aromatic protons (8.52 d, 7.97 t, 7.56 t and 7.11 d). The difference was observed in the aliphatic region, where a deshielded quartet methylene (δ_H 2.40) and triplet methyl groups (δ_H 1.12) were observed in place of the methyl singlet in 2, suggesting that propamide replaced acetamide. This was consistent with a molecular formula of C₁₀H₁₁NO₃ established by a negative HR-ESITOFMS experiment (m/z 192.0667, + Δ 1.0 mmu), which added one methylene unit to 2. Further analysis of a 2D NMR data set (Figure 2) supported this assignment, concluding 1 to be *N*-propionylantranilic acid.

Compounds 1 and 2 were synthesized by a reaction between anthranilic acid and Ac₂O or propionyl chloride for structure confirmation and bioactivity evaluation. The physicochemical

Figure 2 Selected HMBC (arrows) correlations supporting the structure of 1. Bold lines denote a spin system connected by COSY correlations.

properties of the synthetic 1 and 2 were essentially the same as those of the natural products.

Compound 1 was for the first time isolated as a natural product, while 2–4 were originally discovered from *Bacillaceae* or *Thermoactinomycetaceae*: 2 from *Bacillus pantothenicus*,¹⁹ 3 from a marine *Bacillus* sp. as an algicide selective to dinoflagellates and raphidophytes and 4 from *B. endophyticus*.²⁰ Isolation of 3 was also reported from *B. endophyticus*,²⁰ an actinomycete *Microbispora aerate*,²¹ *Thermoactinomyces* sp.²⁰ and fungus *Tricladium* sp.²² These metabolites are obviously derived from tryptophan or its biosynthetic intermediate anthranilic acid. Because tryptophan is one of the most energetically expensive amino acids to biosynthesize and hence its abundance in proteins among least,²³ the enhanced production of common tryptophan-related metabolites by kinships of *Bacillaceae* casts a question on their adaptative roles in producers' life history.

In contrast, their involvement in plant physiology is rather relevant, as we see the example of indole-3-acetic acid or auxin.²⁴ Many phytopathogenic and phytostimulating bacteria can produce this plant hormone for their successful colonization to the hosts, and all of the biosynthetic pathways identified to date are mapped downstream of anthranilic acid or tryptophan. In a previous study, significant inhibition of root growth was observed by treating cress with 1000 p.p.m. of 2, 5 or tryptophan.²⁵ In addition, *N*-acetyl propargyl anthranilate applied at a rate of 50 pound per acre (= 56 kg ha⁻¹) completely suppressed broadleaf beans.²⁶ To our disappointment, however, 1 exhibited only marginal inhibition against a rice field weed *Echinochloa crus-galli* at 10 p.p.m. before and after germination. Activity against *Scirpus juncooides* was only detectable when the seeds were pretreated with 10 p.p.m. of 1 and 2.

EXPERIMENTAL PROCEDURE

General

The optimal growth temperature was determined using a TN-2148 temperature gradient incubator (Advantec Toyo Kaisha, Ltd., Tokyo, Japan). UV spectrum was recorded on a U-3210 spectrophotometer (Hitachi High-Tech Technologies, Co., Tokyo, Japan). IR spectrum was measured on a Spectrum 100 (Perkin-Elmer Inc., Waltham, MA, USA). NMR spectra were obtained on an AVANCE 400 or an AVANCE 500 spectrometer (Bruker, Billerica, MA, USA). HR-ESITOFMS were recorded on a Bruker micrOTOF mass spectrometer. Cosmosil 75C18-PREP (Nacalai Tesque, Inc., Kyoto, Japan, 75 μ m) was used for ODS flash chromatography.

Microorganism

The algal specimen, identified as some *Spirogyra* species (phylum *Chlorophyta*) based on the colony morphology, was collected in an irrigation ditch in Imizu, Toyama, Japan, in December 2009. The alga was kept in a sterile polypropylene tube (50 ml) and immediately brought to the laboratory. A piece (500 μ l) taken from the specimen using scissors and tweezers was placed in a plastic tube (15 ml) containing 1 ml of autoclaved distilled water and ground with a

sterilized glass pestle. After the major contents were sedimented, an aliquot of the supernatant (50 µl) was spread over ISP-2G solid medium, which is composed of glucose 0.4%, malt extract (Difco Laboratories, Detroit, MI, USA) 1%, yeast extract (Difco Laboratories) 0.4%, calcium chloride dehydrate 0.04%, gellan gum 0.7% with pH adjusted at 7.3. The plate, enclosed in a polyethylene bag to avoid loss of water, was incubated at 50 °C. The medium surface was mostly covered by mucous bacterial layers, but a grayish white powdery colony was observed after overnight incubation. This was picked on Bn-2 agar (soluble starch 0.5%, glucose 0.5%, meat extract (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) 0.1%, yeast extract (Difco Laboratories) 0.1%, NZ-case (Wako Pure Chemical Industries, Ltd., Osaka, Japan) 0.2%, NaCl 0.2%, CaCO₃ 0.1%, agar 1.5%) and then repeatedly transferred onto the same medium until the contaminants were removed. The isolate, coded IT-2L, was subjected to phylogenetic analysis based on 16S rRNA gene sequence by the method described previously.²⁷ For a 1425-bp nucleotide sequence, thus, amplified was searched the closest recognized relative in the DDBJ database using BLAST algorithm, which retrieved *Laceyella sacchari* (GenBank accession no. EU430566) with 99% identity.

Temperature gradient test

Strain IT-2L grown on Bn-2 agar medium was inoculated on slant Bn-2 agar medium in 18 mm test tubes. The cultures were incubated in a temperature gradient from 25 to 60 °C at 1.46 °C intervals. At hour 12, 24 and 48, their growth was carefully observed and scored based on the indices defined as follows: 0, no growth; 1, substrate mycelia observed; 2, aerial mycelia formed sparsely on substrate mycelia; 3, aerial mycelia covered 1/3 of substrate mycelia; 4, aerial mycelia covered 1/2 of substrate mycelia; 5, aerial mycelia covered all part of substrate mycelia. Cultures incubated at 49.8–52.7 °C for 12 h and at 46.9–52.7 °C for 24 h scored the highest (4 and 5, respectively), suggesting that the optimal growth temperature of IT-2L is 50–53 °C.

Fermentation

Strain IT-2L cultured on a Bn-2 agar medium was inoculated into test tubes each containing 20 ml of the V-22 seed medium (pH 7.0) consisting of 1% soluble starch, 0.5% glucose, 0.3% NZ-case, 0.2% yeast extract, 0.5% Tryptone (Difco Laboratories), 0.1% K₂HPO₄, 0.05% MgSO₄ 7H₂O and 0.3% CaCO₃. The test tubes were cultivated on a shaker (120 r.p.m.) at 50 °C for 24 h. The seed culture (20 ml) was transferred into 21 flasks each containing 500 ml of the A-11M production medium (pH 7.0) containing 2.5% soluble starch, 0.2% glucose, 0.5% yeast extract, 0.5% polypeptone (Wako Pure Chemical Industries), 0.5% NZ-amine (Wako Pure Chemical Industries), 0.3% CaCO₃ and 1% Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan). The inoculated flasks were cultured on a rotary shaker (200 r.p.m.) at 50 °C for 4 days.

Extraction and isolation

At the end of the fermentation period, 250 ml of 1-butanol was added to each flask, and they were allowed to shake for 1 h. The mixture was centrifuged at 6000 r.p.m. for 10 min, and the organic layer was separated from the aqueous layer containing the mycelium. Evaporation of the solvent gave approximately 1.3 g of the extract from a 5 l-culture. The crude extract (1.3 g) was subjected to silica gel column chromatography with a step gradient of CHCl₃/MeOH (1:0, 20:1, 10:1, 4:1, 2:1, 1:1 and 0:1 v/v). Fraction 3 was concentrated to provide 481 mg of brown oil, which was further purified by reversed-phase HPLC (Cosmosil AR-II10 × 250 mm, Nacalai Tesque). Using aqueous MeCN containing 2% formic acid as a solvent, a gradient elution program of 15 to 40% MeCN for 0–15 min; 40 to 85% for 15–25 min, 85% for 25–32 min, 85 to 15% for 32–35 min at a flow rate of 4.0 ml min⁻¹ yielded *N*-acetylthranilic acid (2, 9.5 mg), *N*-acetyltryptamine (5, 35.1 mg), *N*-propionylthranilic acid (1, 2.7 mg), bacillamide B (4, 3.7 mg) and bacillamide A (3, 20.8 mg) with retention times of 11.3, 12.8, 14.7, 17.6 and 21.2 min, respectively.

N-propionylthranilic acid (1)

Colorless needle: UV (MeOH) λ_{max} nm (ε) 305 (4700), 252 (14 000), 221 (28 000). IR (ATR) ν_{max} (cm⁻¹) 1673, 1585, 1518, 1448, 1293, 752. HR-ESI-TOFMS: 192.0667 ([M-H]⁻, C₁₀H₁₁NO₃; calc. 192.0664).

Synthesis of 1 and 2

To a solution of anthranilic acid (1.4 g, 10 mmol) in dry pyridine (1.6 ml) was added either Ac₂O (1.13 ml, 12 mmol) for the synthesis of 1 or propionyl chloride (1 ml, 11 mmol) for the synthesis of 2 at 0 °C. After stirring for 24 h at an ambient temperature, the reaction mixture was concentrated under reduced pressure, and the residue was purified by ODS flash chromatography with a stepwise elution using aqueous MeCN containing 0.05% TFA from 0:1 or 3:7 to 9:1 to give 1 (1.13 g, 80.6% yield) or 2 (516 mg, 36.9% yield), respectively.

Germination herbicide test

A seed of *Scirpus juncoides* or *E. crus-galli* was soaked in plant tissue culture medium (10 mm depth) for 24 h before the treatment. A test solution, prepared by dissolving the compound in an emulsifier followed by dilution with water, was added to the seed culture, and the test tubes were maintained at 30 °C under continuous illumination. Six days after the treatment, herbicidal activity was evaluated by scoring the progress of germination relative to the untreated group according to the criteria defined as follows: 5, germination completely inhibited; 4, only a slight sign of germination was observed; 3, progress of germination was mostly (= 70–89%) inhibited; 2, the progress of germination was around 40–69% of the control; 1, germination was inhibited but only to a limited extent; 0, germination was not inhibited at all. Compound 1 at 10 p.p.m. scored 1 against both the plants, whereas compound 2 at the same concentration was only effective to *S. juncoides* as scored 1.

Seedling growth inhibition test

The test procedure was essentially the same as that described in the previous section except for the use of one-leaf stage seedlings and 48 mm test tubes filled with culture medium to 40 mm depth. Following criteria were adopted to score the herbicidal activity of the agents: 5, growth completely inhibited; 4, only a slight sign of growth was observed; 3, growth was mostly (= 70–89%) inhibited; 2, the size of the seedlings was about the half of the control plants; 1, growth was partly inhibited; 0, growth was not inhibited at all. Compound 1 at 10 p.p.m. scored 1 against *E. crus-galli*.

ACKNOWLEDGEMENTS

This work is partly supported by Grant-in-aid for Scientific Research (grant 23710259) to N. O. from the Ministry of Education, Culture, Sports and Technology of Japan.

- 1 Van Epps, H. L. René Dubos: unearthing antibiotics. *J. Exp. Med.* **203**, 259 (2006).
- 2 Demain, A. L. & Sanchez, S. Microbial drug discovery: 80 years of progress. *J. Antibiot.* **62**, 5–16 (2009).
- 3 Wilson, Z. E. & Brinble, M. A. Molecules derived from the extremes of life. *Nat. Prod. Rep.* **26**, 44–71 (2009).
- 4 Pettit, R. K. Culturability and secondary metabolite diversity of extreme microbes: expanding contribution of deep sea and deep-sea vent microbes to natural product discovery. *Mar. Biotechnol.* **13**, 1–11 (2011).
- 5 Yoon, J. H. & Park, Y. H. Phylogenetic analysis of the genus *Thermoactinomyces* based on 16S rDNA sequences. *Int. J. Syst. Evol. Microbiol.* **50**, 1081–1086 (2000).
- 6 Craveri, R., Coronelli, C., Pagani, H. & Sensi, P. Thermorubin, a new antibiotic from a thermoactinomycete. *Clin. Med.* **71**, 511–521 (1964).
- 7 Johnson, F. *et al.* Thermorubin 1. Structure studies. *J. Am. Chem. Soc.* **102**, 5580–5585 (1980).
- 8 Isaac, B. G., Ayer, S. W., Letendre, L. J. & Stonard, R. J. Herbicidal nucleosides from microbial sources. *J. Antibiot.* **44**, 729–732 (1991).
- 9 Hafenbradl, D. *et al.* Sibyllimycine 5,6,7,8-Tetrahydro-3-methyl-8-oxo-4-azaindolizidine, a Novel Metabolite from *Thermoactinomyces* sp. †*Angew. Chem. Int. Ed.* **35**, 545–547 (1996).
- 10 Osada, M. & Kamimura, M. (Sapporo Breweries Ltd.), BA-12, from *Thermoactinomyces* as a smut-controlling agent. JP02073046 (1990).
- 11 Kanoh, H. *et al.* Mechercharmycins A and B, cytotoxic substances from marine-derived *Thermoactinomyces* sp. YM3-251. *J. Antibiot.* **58**, 289–292 (2005).
- 12 Matsuo, Y. *et al.* Urukthapelstatin A, a novel cytotoxic substance from marine-derived *Mechercharmyces asporophorigenens* YM11-542. I. Fermentation, isolation and biological activities. *J. Antibiot.* **60**, 251–255 (2007).
- 13 Ōmura, S., Suzuki, Y., Kitao, C., Takahashi, Y. & Konda, Y. Isolation of a new sulfur-containing basic substance from a *Thermoactinomyces* species. *J. Antibiot.* **28**, 609–610 (1975).
- 14 Konda, Y., Suzuki, Y., Omura, S. & Onda, M. Alkaloid from *Thermoactinomyces* species. *Chem. Pharm. Bull.* **24**, 92–96 (1976).

- 15 Jeong, S., Ishida, K., Ito, Y., Okada, S. & Murakami, M. Bacillamide, a novel algicide from the marine bacterium, *Bacillus* sp. SY-1, against the harmful dinoflagellate, *Cochlodinium polykrikoides*. *Tetrahedron Lett.* **44**, 8005–8007 (2003).
- 16 KorKmaz, Ç. A., Hameş-Kocabaş, E. E., Uzel, A. & Bedir, E. Tryptamine derived amides with thiazole ring system from *Thermoactinomyces* strain TA66-2. *Magn. Reson. Chem.* **46**, 80–83 (2008).
- 17 Lacey, J. *Thermoactinomyces sacchari* sp. nov., a thermophilic actinomycete causing bagassosis. *J. Gen. Microbiol.* **66**, 327–338 (1971).
- 18 Pouchert, J. C. & Behnke, J. *The Aldrich Library of ¹³C and ¹H FT NMR Spectra*. 1st edn, Vol 2, 1362 (Aldrich Chemical Company, Inc, USA, 1993).
- 19 Gebhardt, K. *et al.* Screening for biologically active metabolites with endo-symbiotic bacilli isolated from arthropods. *FEMS Microbiol. Lett.* **217**, 199–205 (2002).
- 20 Socha, A. M., Long, R. A. & Rowley, D. C. Bacillamides from a hypersaline microbial mat bacterium. *J. Nat. Prod.* **70**, 1793–1795 (2007).
- 21 Ivanova, V. *et al.* Microbiaeratin, a new natural indole alkaloid from a *Microbispora aerata* strain, isolated from Livingston Island, Antarctica. *Prep. Biochem. Biotechnol.* **37**, 161–168 (2007).
- 22 Zou, X. Two new imidazolone-containing alkaloids and further metabolites from the ascomycete fungus *Tricladium* sp. *Chem. Biodiv.* **8**, 1914–1920 (2011).
- 23 Akashi, H. & Gojobori, T. Metabolic efficiency and amino acid composition in the proteomes of *Escherichia coli* and *Bacillus subtilis*. *Proc. Natl Acad. Sci.* **99**, 3695–3700 (2002).
- 24 Spaepen, S., Vanderleyden, J. & Remans, R. Indole-3-acetic acid in microbial and microorganism-plant signaling. *FEMS Microbiol. Rev.* **31**, 425–448 (2007).
- 25 Audus, L. J. & Quastel, J. H. Toxic effects of amino-acids and amines on seedling growth. *Nature* **4059**, 222–223 (1947).
- 26 Pawloski, C. E. (Dow Chemical Co.), 2-Propynyl ester of *N*-acetyl anthranilic acid, US3636078 (1972).
- 27 Katsuta, A., Adachi, K., Matsuda, S., Shizuri, Y. & Kasai, H. *Ferrimonas marina* sp. Nov. *Int. J. Syst. Evol. Microbiol.* **55**, 1851–1855 (2005).